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Renal proximal tubular epithelium from patients with nephropathic cystinosis: Immortalized cell lines as *in vitro* model systems

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Renal proximal tubular epithelium from patients with nephropathic cystinosis: Immortalized cell lines as *in vitro* model systems. The renal proximal tubule is a major site of injury in a variety of congenital/metabolic diseases including nephropathic cystinosis, the most commonly known cause of renal Fanconi's syndrome. In this lysosomal storage disease there are defects in proximal tubule function within the first few months of life. While culture of renal tubular cells from the urine of these patients is possible, development of immortalized cell lines would insure large numbers of homogeneous cells for studies of renal epithelial cell morphology and pathophysiology in this disease. To develop immortalized cells, cystinotic and normal proximal tubular cells in culture were exposed to an immortalizing vector, containing pZiptsU19 with the temperature sensitive SV40 T-antigen allele tsA58U19 and a neomycin resistance gene, and neomycin-resistant tubular cells were selected for propagation. Ten clones from cystinotic patients have been developed and characterized. All clones express T-antigen at permissive temperature (33°C). Immortalized cells have an epithelial morphology and grow to form confluent monolayers; doubling times vary from 31 to 86 hours. Cystinotic clones are keratin, MDR P-glycoprotein, and alpha-95 kD brush-border associated protein positive but Tamm-Horsfall protein negative by immunocytochemistry, as are normal proximal tubule cells immortalized with this vector. This is consistent with a proximal tubule origin of the cystinotic clones. The cystine content of the cystinotic cells is 70 to 160 times that of normal renal proximal tubular cells in culture, with most of the cystine sequestered in cell lysosomes, confirming that these cell lines express the storage defect. Lysosomes from representative cell lines give a dense lysosome fraction upon Percoll gradient centrifugation, making it possible to purify lysosomes from these cells with no significant contamination by other organelles. These immortalized cystinotic human renal tubular epithelial cell lines should serve as a useful *in vitro* model for the study of tubular epithelial cell and lysosomal abnormalities in cystinosis, enabling definition of pathogenesis of tubular cell dysfunction in this prototypic disease.

Many disease processes affect the tubular epithelial cells of the human kidney. *In vitro* model systems are available for the study of extrinsic injury to these epithelia [1–3], and recent progress has been made in developing human renal immortalized tubular cell lines from kidneys of normal individuals [4–6]. However, few model systems exist for the many relatively rare hereditary and metabolic disorders that cause early and significant renal tubular epithelial cell dysfunction and, in many cases, ultimate renal failure. Few animal models for these rare diseases are presently available and our knowledge of the cellular and molecular mechanisms of these diseases, while substantial, has been derived primarily from fibroblasts and/or leukocytes from affected individuals. Renal epithelial cell systems from these individuals would be very useful in defining cell pathology and pathophysiogical mechanisms involved.

We have a particular interest in defining renal tubular cell pathology in nephropathic cystinosis. This disease is an autosomal recessive disorder in which cystine accumulates in lysosomes due to a defect in a lysosomal membrane cystine transporter [7]. The earliest manifestation of this disease is renal tubular Fanconi's syndrome, beginning within the first six months of life and leading to major nutritional deficits, with ultimate progression to renal failure by the second to third decade [8]. Early treatment with cysteamine, a thiol compound which depletes lysosomes of stored cystine [9], markedly extends renal survival [10]. Very early treatment of these patients with cysteamine may also prevent Fanconi's syndrome, but intervention must begin very early in life and is not always successful [10]. Moreover, many patients are only diagnosed after defects in renal tubular absorption have already developed.

Attempts have been made to develop model systems for nephropathic cystinosis, but most of these have relied upon acute cell loading with cystine via administration of cystine-dimethyl ester *in vitro*, an experimental setting which is not completely analogous to the cell injury seen in this storage disorder. Since we have had success in culturing human renal epithelial cells from the urine of subjects with normal kidneys [11], we attempted to culture renal tubular cells from the urine of young patients with cystinosis who had not yet suffered renal failure, as a benign and non-invasive way of obtaining these mutant epithelial cells for study. We have had substantial success with this approach, having retrieved and cultured epithelial cells from the urine of a significant number of these patients. Upon characterization of these cells, many were shown to have the morphology and histochemical properties of cells of renal proximal tubular origin [12].

However, since primary culture systems have a limited life span *in vitro* and can undergo only a limited number of passages, there

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are difficulties in utilizing such cultures on an ongoing basis for controlled studies. Moreover, repeated harvesting is tedious and time consuming. Despite strategies including a mild salicylate challenge, cell numbers obtained from each isolate are relatively small, and the cell population obtained in this way is potentially heterogeneous. Therefore, we decided to develop immortalized clonal cell lines from stores of cryopreserved renal cells from patients with cystinosis. We now describe the development and characterization of a number of clones of immortalized renal tubular cells from two of these patients. These cell lines express differentiation markers consistent with proximal tubular origin, and express the lysosomal cystine accumulation which is characteristic of the cystinsosis phenotype. Analysis of cell organelles from a number of these clones on Percoll gradient centrifugation reveals a large dense lysosome fraction apparently free of significant contamination from other organelles. These cell lines represent the first available *in vitro* system for study of epithelial cells in this disorder.

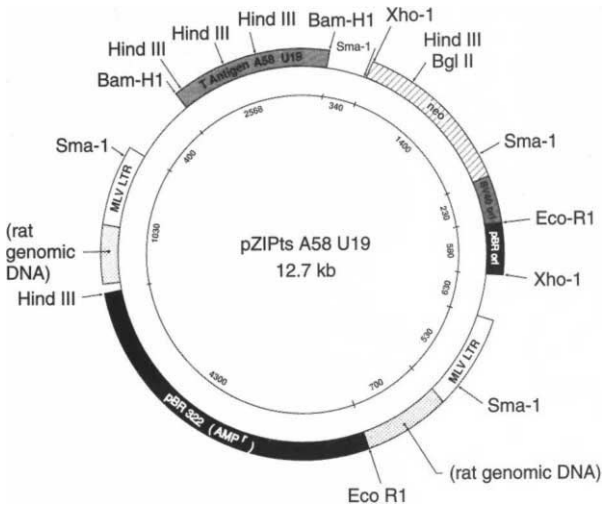

Fig. 1. *Schematic of the recombinant retrovirus plasmid pZIPneoTA58U19 used to construct the immortalizing vector.* The construct contains an SV40 large T antigen segment containing tsA58 and U19 mutations, conferring temperature sensitivity and enhanced transfection efficiency. It also contains a neomycin resistance gene (neo) and a Modified Moloney Murine Leukemia Virus long terminal repeat (MMLV-LTR). The construct was a gift of Dr. R. McKay.

Methods

As described in our previous report [12], clean catch 24-hour urine specimens were obtained from two patients with nephropathic cystinosis being evaluated at the NIH Clinical Research Center per a protocol approved by institutional review boards at the Johns Hopkins University School of Medicine and at the NIH. Specimens were refrigerated until processed. Urine aliquots were centrifuged at 72 × g for five minutes, and the pellets resuspended in culture medium and seeded into culture vessels. Cells were grown in basal medium, a 1:1 ratio of Hams F12:DMEM (Dulbecco's Modified Essential Medium) with 25 mM Hepes, 10% FBS, 50 μ/ml penicillin, 50 μg/ml streptomycin, and 2.5 μg/ml Amphotericin. Cells were propagated, passaged, and then frozen at −80°C in basal medium + 10% DMSO until retrieval for immortalization.

For development of the immortalizing recombinant retrovirus, the plasmid pZipneoTA58U19 was used; this plasmid has been used previously to immortalize human CNS precursor cells [13]. The T-antigen segment is the *Bgl*I (5234) to *Hpa*I (2666) fragment of SV40 containing both the ts A58 (conferring temperature sensitivity [14]), and U19 (enhancing transfection efficiency [15]) mutations. The construct also contains a neomycin resistance gene and a modified Moloney Murine Leukemia Virus long terminal repeat (Fig. 1). The construct was confirmed by restriction mapping, and the plasmid was then transfected into the psi-CRIP amphotropic packaging cell line [16].

PZipneoTA58U19 was introduced into psi-CRIP cells using calcium phosphate coprecipitation. After overnight transfection, cells were placed in fresh basal medium for 48 hours, and clones were then selected in the presence of 1 mg/ml geneticin (G418). Resistant colonies were harvested and propagated, and supernatants from them titered in cultures of 3T3 cells [16]. Briefly, supernatants were serially diluted in basal medium and introduced into culture vessels in the presence of polybrene (6 μg/ml). After overnight incubation, the supernatants were removed and replaced by basal medium containing G418. After 8 to 10 days, resistant colonies were counted and colony-forming units calculated. Two clones, PZA 11 and PZA 13, produced supernatants containing 4×10^4 and 3.6×10^5 colony forming U/ml, respectively, which were then used to immortalize cultured cystinotic and normal renal tubular cells. Supernatants were flash frozen in liquid nitrogen and stored at −80°C until use.

Cryopreserved cystinotic cells cultured from isolates from two unrelated patients (CYS 60 and CYS 92) were retrieved from cyropreservation and seeded in basal culture medium into 35 mm culture plates (Falcon; 1×10^5 cells/plate). Supernatants from PZA 11 and PZA 13 were introduced with polybrene for a 24 hour incubation. The supernatant was then removed and neomycin selection was begun with G418 at 1 mg/ml in basal medium. At 8 to 10 days, well-spaced resistant colonies were trypsizined from the growth surfaces, transferred to individual wells in 24-well plates, fed and monitored for attachment and growth. A number of clones were developed from each isolate and propagated at 33°C (permissive temperature).

Normal human proximal tubule epithelia were obtained by explant culture of kidney cortex from normal human nephrectomy specimens plated in serum-free medium supplemented with specific growth factors previously shown to be selective from growth and differentiation of proximal straight tubule cells [1, 17]. Rapidly proliferating epithelia were treated with pZA supernatants as described above and G418 resistant cells were cloned by limiting dilution. Origin from proximal straight tubule of the clones of immortalized cells used for these studies was confirmed by marker analysis, for alkaline phosphatase, aminopeptidase N, CHIP-28, and MDR (P. Wilson, data not shown).

Clones of immortalized cystinotic cells were subsequently characterized. Cell morphology in culture and growth kinetics, measured as doubling times calculated during exponential growth phase, were monitored. Clones were also assessed for expression of T-antigen using a murine monoclonal antibody to large T-antigen and an indirect immunofluorescence technique; slides were visualized using a Zeiss fluorescence microscope. For assessment of cellular differentiation, cystinotic cells were seeded on

Matrigel, and grown to confluence at 39°C (non-permissive temperature) for six days in Hams F12:DMEM containing 2.5% serum with hormonal supplmentation (2.5 μg/ml insulin, 0.05 μg/ml hydrocortisone, 5 μg/ml transferrin; 5 ng/ml sodium selenite and 20 ng/ml triiodothyronine). They were then stained for various proximal and distal nephron markers using a streptavidin biotin immunoperoxidase technique (Vector Laboratories [18]) with primary antibodies against cytokeratin (epithelium), Tamm-Horsfall protein (distal tubule), and MDR P-glycoprotein and alpha-95 kD brush border protein (proximal tubule). Positive controls were frozen sections of human kidney and normal human kidney cells immortalized with the same recombinant vector selected with G418 and cloned; negative controls were monolayers stained following omission of the primary antibody or stained following exposure to non-immune serum.

Cystine levels were measured on whole cell suspensions and on granular fractions from each cystinotic cell line and from normal controls within five days of reaching confluence. Whole cell cystine was also determined in one normal and two cystinotic clones maintained at confluence for four weeks, to assess influence of incubation time on cystine accumulation. Isolation of the granular fraction, enriched in lysosomes, was prepared from cultures grown to confluence at 37°C in four 100 mm^2 Primaria dishes (Falcon) in the presence of Hams F12:DMEM with 2% FBS and media supplements (see above). Cultures were placed on ice, then washed and harvested by scraping into 0.25 M sucrose, 5 mM EDTA, pH 7.0 (sucrose/EDTA) and sedimented briefly at 70 g. For measurements of total cell cystine levels, cell pellets from two dishes were resuspended in 5 × volume of 10 mM N-(ethyl)maleimide in 10 mM sodium phosphate buffer (NEM/PO_4). For isolation of the granular fraction, pelleted cells from the remaining two dishes were resuspended to 5 × volume in sucrose/EDTA containing proteinase inhibitors (2 mM phenylmethyl sulfonyl fluoride, 10 μM pepstatin, 1 μg/ml leupeptin and 1 μg/ml aprotinin). Cells were disrupted by aspiration through a Pasteur pipette, and cell rupture monitored by microscopic observation of nuclear release. Nuclei and cellular debris were sedimented at 200 × g for 10 minutes. The supernatant containing organelles, plasma membrane vesicles and soluble protein was then centrifuged at 20,000 × g for 10 minutes, and the pellet, resuspended in a minimum volume of NEM/PO_4 with proteinase inhibitors (see above), was taken as the granular fraction enriched in lysosomes. β-hexosaminidase was measured fluorometrically by the cleavage of 4-methylumbeliferyl, 4-acetyl-β-D-glucosaminide using the method of Fisher et al [19]. Cystine levels in total cell and granular fractions were determined using a bioassay method [20], with results standardized as nmoles 1/2 cystine (cysteine) per mg protein. Protein was determined using the BCA reagent. (Pierce). Recovery of β-hexosamidase and cystine in granular fractions were calculated as a percent of the total values measured in the corresponding whole cell suspension.

Lysosomal vesicle isolation

Lysosomes were isolated from 5 × 10^7 normal human and cystinotic renal cells essentially as described above in a 0.25 M sucrose, 5 mM EDTA buffer, pH 7.0 containing proteinase inhibitors (2 mM phenylmethylsulfonyl fluoride, 10 μM pepstatin, 1 μg/ml leupeptin, and 1 μg/ml aprotinin). Lysosomes were further separated from other cellular organelles in the granular fraction on a 25% Percoll density gradient (Pharmacia) and 0.5 ml fractions collected. Aliquots of each step in the purification and each fraction from the Percoll gradient were assayed for protein content, for alkaline phosphatase activity to localize plasma membrane vesicles, for β-hexosaminidase as a measure of lysosomal integrity, and for β-glucosidase as a marker for lysosomal membranes. Alkaline phosphatase was assayed spectrophotometrically by the cleavage of p-nitrophenylphosphate [21] in 50 mM glycine, pH 10.5, containing 0.25 mM $MgCl_2$, 2.5 mM zinc acetate, and 5 mM $CaCl_2$. Reactions were stopped with the addition of 5 N NaOH and p-nitrophenol (PNP) was used as standard. β-hexosaminidase activity [19] was used to track purification of intact lysosomes as described above. β-glucosidase was assayed by the hydrolysis of 4-methylumbelliferyl β-D-glucoside [22] in 0.1 M KPO_4, pH 5.9, with 0.25% sodium taurocholate and 0.15% Triton X-100; 0.2 M glycine, pH 10, was used to stop the reactions. The fluorescent product, methylumbelliferone (MeU) was used to standardize both β-hexosaminidase and β-glucosidase activity. Protein was estimated with the Bradford reagent (Bio-Rad) with BSA as standard.

In a refinement of these methods, 1 mM $CaCl_2$ was added to granular fractions prior to Percoll gradient centrifugation in order to decrease the buoyant density of the mitochondria, which could contaminate the dense lysosome peak. Assays were performed for α-mannosidase (Golgi vesicles) and cytochrome C oxidase (mitochondria) as described by Storrie and Madden [23]. Alpha-mannosidase activity was measured by the cleavage of 4-methylumbelliferyl-α-D-mannopyranoside in (Dulbecco's) PBS. Cytochrome C oxidase activity of Percoll fractions was measured spectrophotometrically as a decrease in absorbance at 550 nm in the presence of reduced cytochrome C in PBS.

Reagents. Hams F12:DMEM, fetal bovine serum, and antibiotics were from Gibco Laboratories (Grand Island, NY, USA). Matrigel was purchased from Beckton Dickinson Laboratories (Bedford, MA, USA). All other chemicals and media supplements were obtained from Sigma Chemical Company (St. Louis, MO, USA). The plasmid pZIPNeoTA58U19 was a gift from Dr. R. McKay, (MIT) and the psi-CRIP cell line was provided by Dr. R.C. Mulligan (MIT). Murine monoclonal antibody 419 to SV40 TAg was a gift from Dr. Thomas Kelly (JHMI); antibody to MDR P-glycoprotein was obtained from Dr. Suresh Ambudkar (JHMI). Cytokeratin antibody was obtained from Biomedical Technology, Inc. (Stoughton, MA, USA) and antibody against Tamm Horsfall protein from The Binding Site (San Diego, CA, USA). Antibody to alpha-95 brush border associated protein was a gift from Dr. M. Farquhar (Yale University). Immunocytochemistry was performed using the Vectastain kit and aminoethylcarbazole from Vector Laboratories, (Burlingame, CA, USA). All reagents for lysosome isolation and enzyme assays were purchased from Sigma Chemical Company.

Results

A total of ten clones of cystinotic renal tubular cells were developed from cystinotic culture isolates CYS 60 and CYS 92 using supernatants from the two psi-CRIP clones (pZA11, pZA13) which had been transfected with pZipneoTA58U19 and which produced high-titer immortalizing supernatants. All cystinotic clones demonstrated a monolayer growth pattern; phase micrographs of three representative clones are shown in Figure 2. Doubling times for all ten clones varied between 31 and 86 hours, at 33°C (permissive temperature; Table 1). Cells from all normal

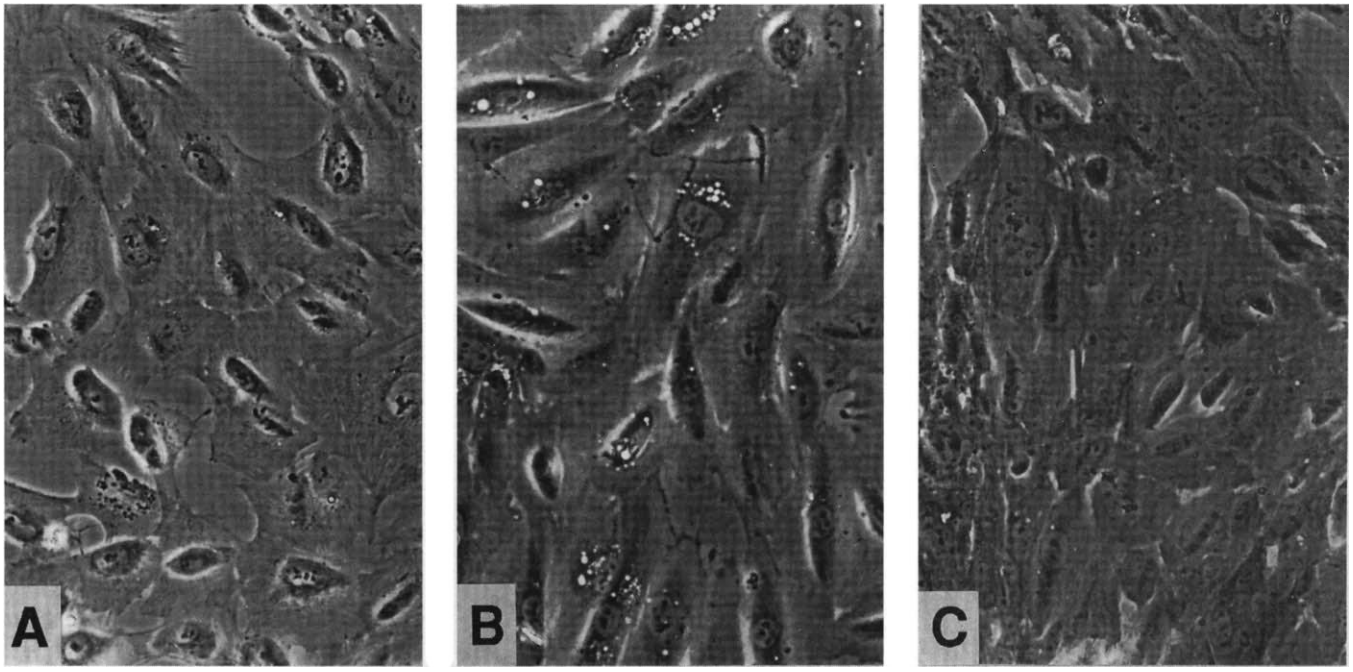

Fig. 2. *Phase contrast micrographs of three representative clones of cystinotic tubular cells photographed at or near confluence.* The cells show a monolayer growth pattern and have polygonal, epithelial cell morphology. (**A.** CYS 60.11.7 (×115); **B.** CYS 60.11.2 (×230); **C.** CYS 92.13.1 (×115).

Table 1. Properties of cystinotic renal tubular cell clones

Clone	Growth	Doubling time *hours*	T ag	Cystine *nmol 1/2 cystine/mg protein*
CYS 60.11.1	Monolayer	47	+[a]	5.74
CYS 60.11.2	Monolayer	48	+	8.18
CYS 60.11.7	Monolayer	86	+	7.55
CYS 92.11.1	Monolayer	38	+	4.50
CYS 92.11.3	Monolayer	31	+	3.25
CYS 92.11.5	Monolayer	38	+	5.70
CYS 92.13.1	Monolayer	50	+	7.31
CYS 92.13.2	Monolayer	44	+	5.01
CYS 92.13.3	Monolayer	41	+	5.79
CYS 92.13.5	Monolayer	46	+	7.99
NHK-PST[b]	Monolayer	48	+	0.06

[a] Positive staining

[b] NHK-PST is a non-clonal pool of normal human kidney proximal tubular cells grown from explant cultures and immortalized using the same recombinant DNA vector used to develop the CYS clonal cell lines.

Table 2. Recovery of granular fraction (G.F.) and whole cell lysosomal β-hexosaminidase and cystine

	Beta-hexosaminidase activity *total nmol Meu*			Total nmol 1/2 cystine		
Culture	G.F.	Whole cell	% Recovery[a]	G.F.	Whole cell	% Recovery
CYS 60.11.1	138	158	87	6.706	6.872	98
CYS 60.11.2	64	151	42	4.606	5.071	91
CYS 60.11.7	59	172	34	2.308	3.101	74
CYS 92.11.1	216	605	43	9.195	9.479	97
CYS 92.11.3	170	455	37	4.914	7.029	70
CYS 92.11.5	235	360	65	5.308	7.481	71
CYS 92.13.1	126	251	50	4.437	5.634	77
CYS 92.13.2	66	209	32	1.976	4.695	42
CYS 92.13.3	107	269	40	4.830	5.695	86
NHK-PST[b]	89	231	35	0.039	0.068	57
NHK-PST	51	118	43	0.052	low	—

[a] (G.F./whole cell) ×100

[b] NHK-PST is a non-clonal pool of normal human kidney proximal tubular cells grown from explant cultures and immortalized using the same recombinant DNA vector used to develop the CYS clonal cell lines.

and cystinotic clones strongly express large T-antigen at 33°C, with some heterogeneity in intensity of staining among cells within each clone. Growth for six days at 39°C reduced but did not eliminate T-antigen staining.

Cells from the cystinotic clones were assayed for cell cystine and results compared to cystine levels in control normal human proximal tubule epithelial cells immortalized with the same supernatants (Table 1). Cell cystine levels of the immortalized normal human proximal tubule (NHK-PST) cells averaged 0.06 nmoles 1/2 cystine/mg protein. These values are nearly identical to those previously determined for normal human renal tubular cells in primary culture [12]. In contrast, the cystinotic clones have comparatively high cystine levels, varying from 3.25 to 8.18 nmoles 1/2 cystine/mg protein. Cell fractionation studies (Table 2) demonstrated that the cell cystine is largely sequestered in the lysosomal fraction; recovery of cystine was proportional to recovery of β-hexosaminidase (a marker of intact lysosomes) in the granular fractions from these cells. These results confirm the basic storage defect in the cystinotic cell lines. Comparison of the clones assayed immediately after reaching confluence and later after four weeks at confluence showed no increase in cystine accumulation with time in culture.

By immunoperoxidase staining, all cells from the immortalized NHK-PST and cystinotic clones were shown to be cytokeratin positive, confirming their epithelial origin (Table 3, and Fig. 3).

Table 3. Expression of differentiation markers by immunocytochemistry in cystinotic renal tubule cell lines

Clone	Keratin	MDR P-glycoprotein	alpha 95 kd brush border protein	Tamm-Horsfall protein
CYS 60.11.1	2-3+	1-2+	2-3+	0
CYS 60.11.2	3+	2+	1+	0
CYS 60.11.7	3+	3+	1-2+	0
CYS 92.11.1	2-3+	1+	2-3+	0
CYS 92.11.3	1+	1-2+	1-2+	0-1+
CYS 92.11.5	1+	1-2+	1-2+	0-1+
CYS 92.13.1	3+	1+	1-2+	0
CYS 92.13.2	1-3+	1+	2-3+	0
CYS 92.13.3	1+	1-2+	1-2+	0
CYS 92.13.5	1+	1+	1-3+	0
NHK-PST	3+	2+	2+	0

Semiquantitative grading (scale 0 to 3+) of immunoperoxidase staining for keratin (epithelium), MDR P-glycoprotein and alpha 95 kd brush border protein (proximal nephron), and Tamm-Horsfall protein (distal nephron).

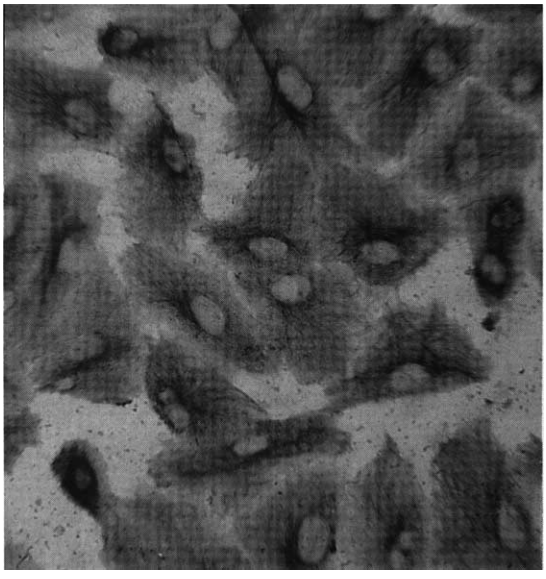

Fig. 3. *ABC-immunoperoxidase stain for cytokeratin, an epithelial cell marker, in a representative cystinotic clone, CYS 92.11.1 (×250).* Note the filamentous intracellular staining.

Results of immunoperoxidase staining for cell expression of a variety of segment-specific antigens are shown in Table 3. Markers included the alpha-95 kD brush-border associated protein and MDR p-glycoprotein, specific for proximal tubule; and Tamm-Horsfall protein, specific for more distal nephron [24]. Clones express markers for proximal tubule comparable to the pattern of staining seen with the normal human proximal tubule cells [1, 24]. However, some heterogeneity in intensity of staining was seen among the cystinotic clones (Table 3). Photomicrographs of monolayers stained for proximal tubule markers (MDR-P-glycoprotein and alpha-95 kD brush border associated protein) in a representative cystinotic clone, CYS 92.11.5, are shown in Figure 4. This clone showed 1 to 2+ staining for both of these antigens.

The results of Percoll density gradient fractionation of representative clones are given in Figure 5. Parts A and B show total β-hexosaminidase (μmol MeU) and total alkaline phosphatase (nmol PNP) activity for one cystinotic clone and one normal clone. Alkaline phosphatase activity, a marker for brush border plasma membrane vesicles, peaked at fractions 2 to 3 from the top of the gradient and became negligible after fraction 6 (Fig. 5 A, B). Assay of β-hexosaminidase activity indicates dense lysosomes are concentrated below fraction 7 (Fig. 5 A, B, C, D). In panels C and D of Figure 5, β-hexosaminidase and β-glucosidase activities are shown. These markers show identical distribution along the Percoll gradient as was expected since both are markers for lysosomes. The cystinotic clones also show a bimodal distribution of lysosomal enzyme markers, indicative of the classic light and dense lysosome populations, present in many normal renal cell lines including LLC-PK_1. The dense lysosomal fraction is free of markers for the Golgi apparatus and for mitochondria, which segregate in fractions 2 to 3 (not shown). Each cystinotic and normal clone displays a reproducible and characteristic pattern of lysosomal marker activity, indicating variable proportions of light and dense lysosomal populations.

Discussion

The cellular defects in a variety of metabolic diseases affecting the renal nephron have not been defined. This is due, in large part, to lack of model systems in which to study these disorders. With few exceptions, there are no true animal models for these diseases. While leukocytes and fibroblasts may be harvested from affected patients for study, these cells largely lack the specialized functions of the epithelial cells impacted by the disease.

Attempts have been made to model nephropathic cystinosis by acute exposure of kidney cells to cystine dimethylester (CDME), a membrane-permeable form of the amino acid which enables rapid cell and lysosome loading with cystine. Using this model, Foreman et al [25] and Salmon and Baum [26] found that CDME loading inhibited volume absorption, transepithelial potential difference, glucose transport, and bicarbonate transport in perfused rodent proximal convoluted tubules. Coor et al subsequently showed that exogenous ATP ameliorated the transport inhibition in this model [27]. Ben-Nun et al acutely loaded cultured LLC-PK_1 cells, a porcine cell line with characteristics of proximal tubule, and found decreased Na,K-ATPase activity and reduction in number and activity of plasma membrane transporters [28]. While these results are intriguing, acute CDME toxicity may not truly model the cellular abnormalities in the human storage disease in which cystine accumulates gradually over time. Also, these systems are obviously not useful for defining the abnormal gene(s) and gene product(s) underlying this storage disease.

To develop model renal epithelial cell primary culture systems which truly express the mutation in nephropathic cystinsosis, we employed a non-invasive strategy which we and others had previously utilized to harvest human renal cells [11, 29–32]. In previous studies [12], we had obtained urine specimens from patients with nephropathic cystinosis, centrifuged the specimens, resuspended the sedimented cells, and placed them in culture vessels. Cell monolayers were obtained from 69% of the specimens which contained renal epithelial cells and which were not contaminated. Fifty-eight percent of these grew to confluent monolayers and could be passaged and/or cryopreserved. Monolayers had an epithelial appearance by phase and electron microscopy and the majority of cells in the monolayers expressed brush

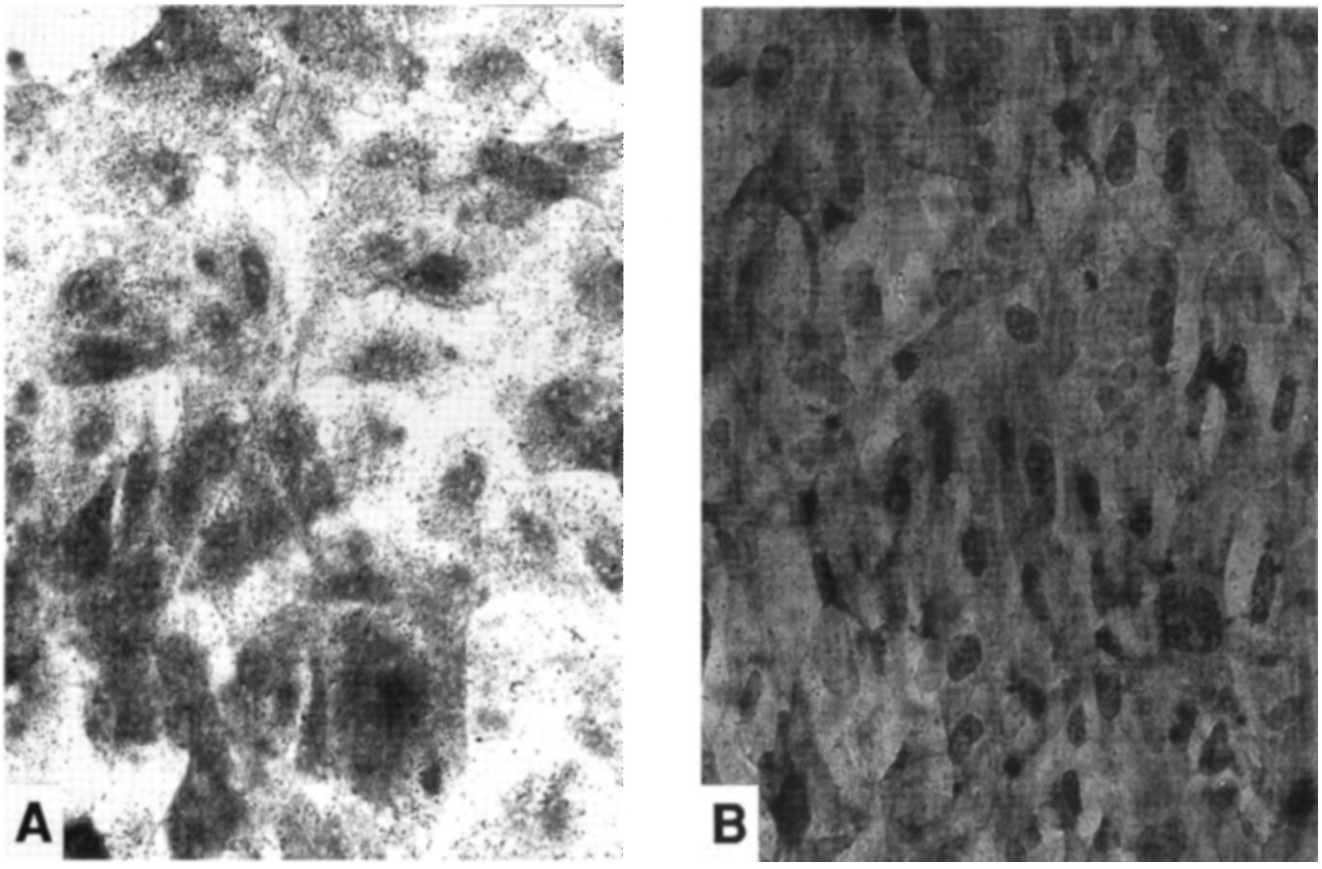

Fig. 4. *ABC-immunoperoxidase stains for alpha 95 kD brush border associated protein* (**A**) *(×400) and MDR P-glycoprotein* (**B**) *(×250) on a representative cystinotic clone, 92.11.5.* Punctate surface staining is clearly seen.

border enzymes typical of renal proximal tubule. These cells also had high levels of intracellular cystine, which could be depleted by exposure to cysteamine.

While many of the epithelial cells cultured from the urine of cystinotic patients originated from the renal proximal tubule, other cells were of more distal origin. However, because of the small numbers of cells it was not possible to purify them and isolate homogeneous proximal (or distal) tubular cells. Even with salicylate challenge, a strategy utilized previously to enhance shedding of renal tubular epithelial [32], cell numbers remained small. Moreover, since cells from one individual may differ from those of another, each isolate must serve as it's own control; cell numbers obtained from each isolate were too small to perform such controlled studies. Lysosomal isolations that could further delineate the membrane transport defect were also not possible. It became necessary to develop immortalized renal epithelial cell lines from these isolates in order to provide the large numbers of mutant cells required for definitive *in vitro* studies.

We had previously developed normal renal epithelial cell lines using an Adeno-12:SV40 hybrid immortalizing virus containing the Adeno EIA and SV40 large T antigens. A number of clones developed with this agent retained characteristics of renal proximal tubule epithelium [4, 5]. However, the Adeno-12-SV40 recombinant lacks a dominant selectable marker enabling early selection of transfected cells, and also has high level constitutive expression of T-antigen. Therefore, for immortalizing cystinotic renal tubular cells, we used a retrovirus construct pZIPneoTA58U19 encoding the mutant SV40 large T-antigen A58U19, which produces a temperature-sensitive expression of large T-antigen, and has the Tn5 neomycin resistance gene as a dominant selectable marker. With the recombinant retrovirus vector generated using this plasmid and the amphotropic packaging line psi CRIP, we have succeeded in developing clonal lines of immortalized cystinotic renal epithelial cells.

Clones of renal tubular cells from two cystinotic patients from different kindreds have been characterized. All clones express markers of proximal tubular cell differentiation, and all express the lysosomal cystine storage defect; normal human renal proximal tubular cell lines immortalized with the same vector have similar properties but lack the storage defect. The levels of cystine contained in these cells (3.25 to 8.18 nmol 1/2 cystine/mg protein) is in the range reported for cystinotic renal tubular cells in primary culture (4.0 ± 0.3 nm/mg) [12, 33] and for cystinotic fibroblasts (6.4 to 9.6 nm/mg) [34], as well as for cystinotic fibroblast cell lines [35]. They are significantly (10- to 40-fold) higher than levels in transformed cystinotic lymphoblasts [34]. These levels are lower than those reported for liver (45.8 to 112.5 nm/mg), pancreas (35.8 nm/mg), and native kidney (16.7 to 101.7 nm/mg) *in situ* [8]; the *in situ* organ cystine levels presumably represent long-term accumulation in tissues with low cell turnover. All cystine levels reported

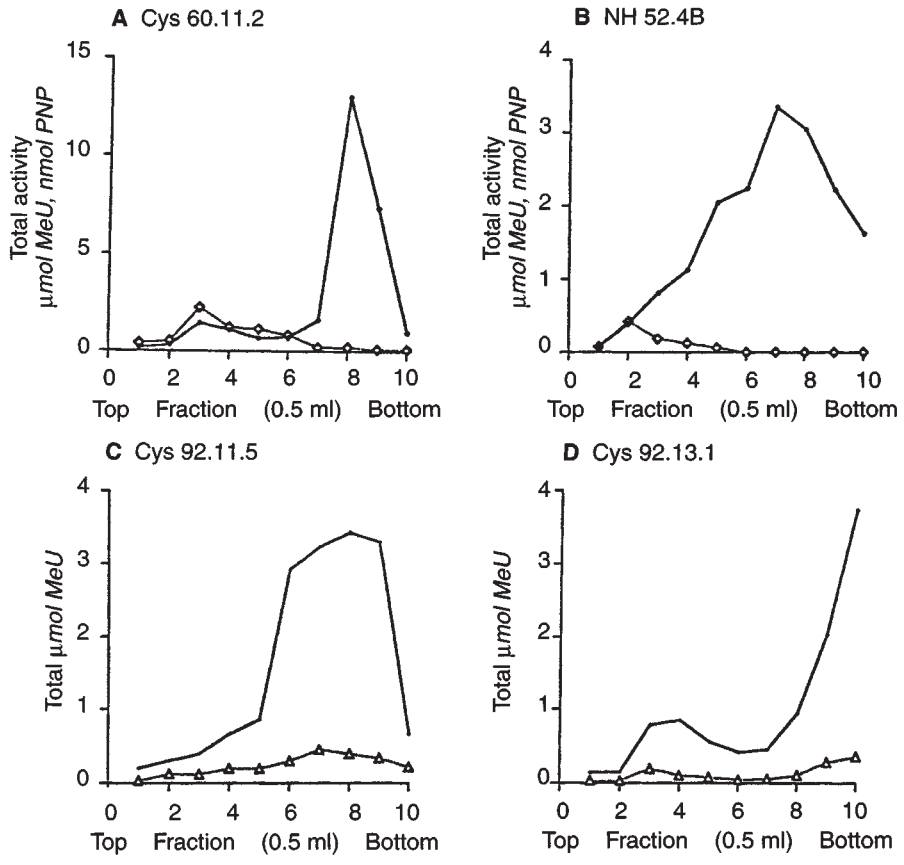

Fig. 5. *Percoll gradient distribution of total alkaline phosphatase activity (◇; nmol PNP); β hexosaminidase (●; μmol MeU); and β-glucosidase (Δ; μmol MeU); recovered in granular fractions prepared from cystinotic* (**A, C, D**) *and normal* (**B**) *proximal tubule cell lines.*

here were performed on confluent monolayers. We have also assessed cystine accumulation over time in stationary monolayer cultures in order to test the hypothesis that cystine accumulation increases over time in cells with low mitotic activity; however, no additional accumulation of cystine was seen. These results are similar to those reported in a cystinotic fibroblast cell line [33]. In addition cell cystine levels in our cultures do not appear to correlate with isolate of origin nor with passage number.

Cystine storage in lysosomes appears to be directly related to the cellular defects in nephropathic cystinosis, since depletion of cell cystine with cysteamine significantly delays and/or alleviates symptoms of the disorder. The cystinotic epithelial cell lines described herein have discrete lysosomal peaks on gradient centrifugation of cell lysates, making it possible to isolate dense lysosomes for studies of physicochemical, structural, and transport properties. While some studies of discrete lysosomal properties have been carried out in cystinotic fibroblasts and leukocytes [36–40], these cells display no known alterations in function in this storage disease. Cystinotic renal epithelial cell lines enable study of lysosomal abnormalities in a systematic fashion in mutant epithelial cells and allow correlation between lysosomal and cellular abnormalities in the disorder.

In summary, renal tubular cell defects are an early manifestation and major cause of morbidity in patients with nephropathic cystinosis. The immortalized renal tubular cell lines we have developed from cystinotic patients express the lysosomal cystine transport defect and should be ideal for the study of the cellular abnormalities underlying this disease. These clones appear to be a unique model for the study of early lysosome abnormalities in cystinosis, and should prove to be a useful tool for defining the molecular pathology in this disorder. Moreover, the general strategy we have employed of urine cell isolation and immortalization should prove useful for developing renal epithelial cell lines from other patients with relatively rare genetic disorders affecting the renal tubule.

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